TRANSGLUTAMINASE AS A BLOOD CLOTTING ENZYME

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Clotting of fibrinogen in vertebrate blood is regulated by two enzymes. First, thrombin converts fibrinogen into fibrin by a process of limited proteolysis in which negatively charged fibrinopeptide fragments, amounting to 3% of the parent protein, are removed (Lorand, 1951 and 1952). This enables the protein, even when present in extremely low concentration, to form ordered molecular aggregates (Bailey et al., 1943; Ferry et al., 1954; Hall and Slayter, 1959; Stryer et al., 1963) at the pH and ionic strength of blood. Subsequently, the fibrin gel is crosslinked through a transpeptidating reaction (Lorand et al., 1962), catalyzed by the thrombin activated fibrin stabilizing factor. Activation of the crosslinking enzyme by thrombin itself (Lorand and Konishi, 1964) provides for synchrony between the hydrolytic and transpeptidating steps (Fig. 1).

Such a "two-enzyme control" (i.e. hydrolysis followed by transpeptidation) was found to hold for all vertebrates examined, including the lamprey eel (Doolittle et al., 1963;

Abbreviations: Tris, tris(hydroxymethylaminomethane); EDTA, ethylenediaminetetraacetic acid; FSF, fibrin stabilizing factor; FSF*, thrombin-activated fibrin stabilizing factor.

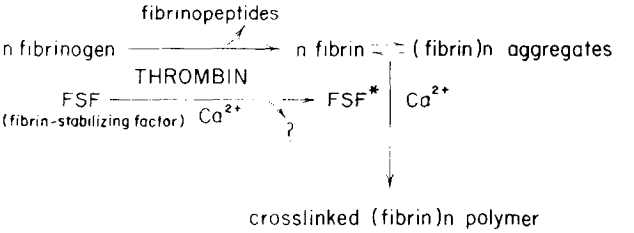

Fig. 1. Illustration of the "two-enzyme control" in the clotting of vertebrate fibrinogen.

Doolittle, 1965; Lorand, 1961). This is in contrast to the situation in Homarus blood where a single enzyme alone (called tissue coagulin, usually isolated from the tail muscle) appears to be responsible for clotting (Glavind, 1948; Duchateau and Florkin, 1954). Moreover, this invertebrate enzyme functions in a manner analogous to the transpeptidase (FSF* in Fig. 1) of vertebrate blood, as seen - for example - by the fact that amines which specifically inhibit only the crosslinking reaction in vertebrates, very effectively inhibit clot formation itself in Homarus plasma (Lorand et al., 1963). This, of course, suggests that crosslinking by transpeptidation is perhaps the original reaction in blood clotting. The rationale of thrombin action on vertebrate fibrinogen would lie mainly in the unmasking (Lorand, 1965; Lorand and Ong, 1966a) of the amine-acceptor sites of the protein by removal of fibrinopeptides. As a result, four side chains - one glutamine and three asparagine residues - per mole of native (bovine) fibrin become available for transpeptidation (Lorand and Ong, 1966b and unpublished data). Homarus fibrinogen does not require such a preparatory unmasking of its crosslinking sites. The thrombin induced conversion of fibrinogen to fibrin seems to be an extra control mechanism developed in vertebrates.

On proposing transpeptidation as a basis for fibrin crosslinking, the similarity between the crosslinking enzyme and another transpeptidase (called transglutaminase), usually purified from guinea pig liver (Clarke et al., 1959), was immediately noted (Lorand et al., 1962). Further studies reinforced the analogy between the two enzymes (Lorand and Jacobsen, 1964; Lorand, 1965; Lorand and Ong, 1966a and b; Loewy et al., 1966), especially in regard to specificity toward amine substrates. Moreover, the similarity also extended to the lobster clotting enzyme (Lorand et al., 1964; Lorand et al., 1965).

In the present paper we show that transglutaminase, purified according to the procedure of Waelsch and Mycek (1962), can replace the thrombin-activated stabilizing factor (FSF* in Fig. 1) for crosslinking bovine fibrin and can also - in lieu of tissue coagulin - rapidly clot lobster blood plasma.

Fig. 2 shows the activity of transglutaminase, as a function of enzyme concentration, on two substrates. The dashed line (triangles; relating to the right-hand ordinate) illustrates the assay of this enzyme by the method of Waelsch and Mycek (1962). A partial digest of casein was incubated with hydroxylamine (at pH 8 and 37°) in the presence of the enzyme exactly as given by these authors, and the hydroxamate formed in ten minutes was determined colorimetrically after the addition of ferric chloride. In Fig. 2, the color yield is expressed in terms of authentic acetohydroxamic acid.

The solid curve illustrates the ability of guinea pig liver transglutaminase to crosslink bovine fibrin. The methodology was quite similar to that previously described (Lorand et al., 1962; Lorand and Jacobsen, 1964). Each reaction mixture contained 0.5 ml of 0.1 M calcium chloride;

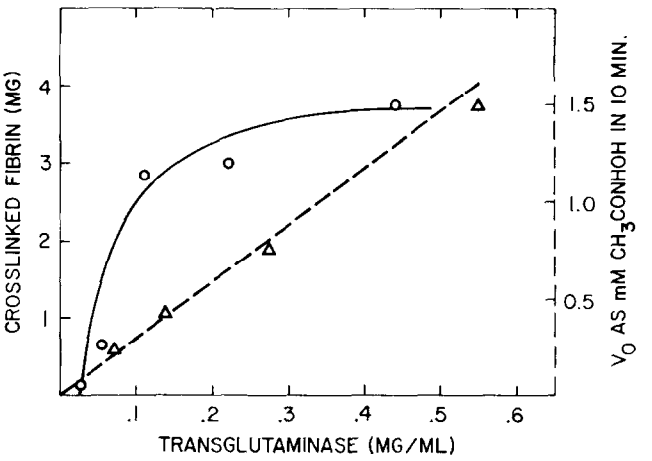

Fig. 2. The crosslinking effect of transglutaminase on bovine fibrin (circles). Assay of the enzyme on casein digest (Waelsch and Mycek, 1962; triangles).

0.5 ml of 0.1 M freshly neutralized glutathione; 1.1 ml of 0.1 N sodium chloride - 0.05 M Tris buffer adjusted to pH 7.5 with hydrochloric acid; 0.1 ml of transglutaminase at various dilutions in the pH 7.5 Tris buffer with 0.01 M EDTA added (see Waelsch and Mycek, 1962); finally, 0.3 ml of 1.5% fibrin solution in 1 M sodium bromide at pH 5.4 (Donnelly et al., 1955). Clot formation occurred in less than one min. Half an hour later, the reaction was terminated by the addition of 2.5 ml of 2% monochloroacetic acid which also dispersed non-crosslinked fibrin. The crosslinked cores of the protein, remaining insoluble in the acid 24 hr later, were estimated (Lorand and Jacobsen, 1964) and are shown on the left-hand ordinate (circles) in Fig. 2.

Just as in the case with the thrombin-activated fibrin stabilizing factor (Konishi and Lorand, 1966), an apparent "threshold concentration" of transglutaminase is required to produce a minimal acid-insoluble crosslinked fibrin in this

test. Also, similar to the activated factor, transglutaminase crosslinks nearly all of the fibrin present (about 90% of the 4.5 mg of fibrin added in the experiments of Fig. 2).

In order to demonstrate that the specific transglutaminase active site of the enzyme preparation was involved in fibrin crosslinking, the latter reaction was also studied in the presence of histamine which is a known competitive substrate of transglutaminase (Clarke et al., 1959). As shown in Fig. 3, histamine inhibited the crosslinking of fibrin by transglutaminase very effectively indeed.

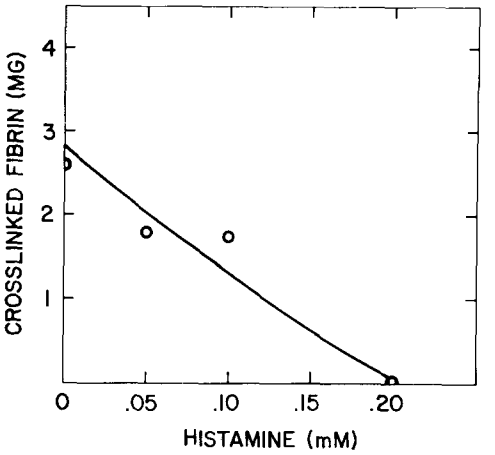

Fig. 3. Histamine inhibition on the crosslinking of bovine fibrin by transglutaminase (0.11 mg/ml).

Perhaps one of the most spectacular protein crosslinking reactions catalyzed by transglutaminase is seen on lobster blood plasma. As presented in Fig. 4, this enzyme can replace the tissue coagulin of lobster itself in a highly potent manner. Clotting times were measured (at 23°) as previously described (Lorand et al., 1963) in mixtures which contained 0.1 ml of 0.2 M calcium chloride, 0.1 ml of μ:15 Tris buffer at pH 7.5, 0.1 ml of transglutaminase (diluted into the Tris buffer with

0.01 M EDTA added) and, finally, 0.2 ml of citrated lobster plasma with a protein content of 10.6 mg. Reciprocal clotting times, with a range of clotting between 10 and 200 sec, were found to be linear with enzyme concentration. No doubt, the ability of transglutaminase to bring about clotting of lobster plasma will provide one of the simplest and most rapid assay systems for measuring the activity of this enzyme.

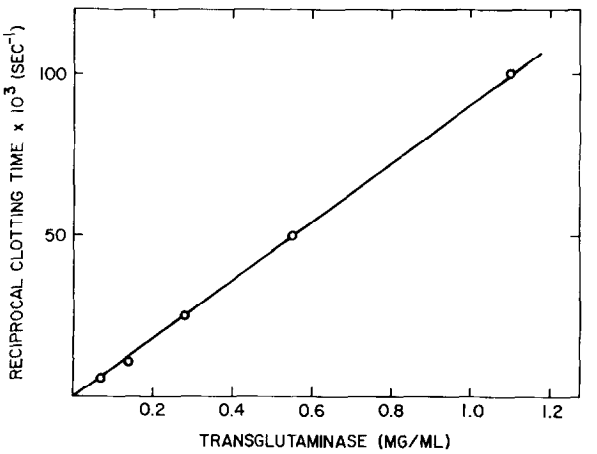

Fig. 4. Clotting of lobster plasma by transglutaminase.

Similar to its effect on crosslinking vertebrate fibrin, the activity of transglutaminase in clotting lobster plasma is effectively inhibited by histamine. With a preparation, which in the absence of histamine was able to clot lobster plasma in 15 sec, clotting times increased to 2 min 20 sec, 8 min 15 sec, and 18 min 20 sec when histamine was added to concentrations of 0.2, 0.4, and 0.6 mM respectively.

The finding that transglutaminase can replace both the crosslinking enzyme of vertebrate fibrin and the coagulin of Homarus, in their specific biological activities, demonstrates extreme similarities among these transpeptidating enzymes.

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